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# Regulation of the Epithelial $\text{Ca}^{2+}$ Channel TRPV5 by the NHE Regulating Factor NHERF2 and the Serum and Glucocorticoid Inducible Kinase Isoforms SGK1 and SGK3 Expressed in *Xenopus* oocytes

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## Key Words

1,25(OH)<sub>2</sub>D<sub>3</sub> • TRPV5 • Calcium transport • Mineralisation • Kidney • Intestine • Bone • IGF1

## Abstract

The epithelial  $\text{Ca}^{2+}$  channel TRPV5 (ECaC1) plays a key role in renal and intestinal  $\text{Ca}^{2+}$  (re)absorption and is thus regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>. The present study aims to explore whether TRPV5 is regulated by the serum and glucocorticoid inducible kinase SGK1, a kinase transcriptionally upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>. To this end cRNA encoding TRPV5 has been injected into *Xenopus* oocytes with or without additional injection of SGK1, its isoforms SGK2 and SGK3, constitutively active S<sup>422D</sup>SGK1, inactive K<sup>127N</sup>SGK1, constitutively active T<sup>308D,S473D</sup>PKB and/or the Na<sup>+</sup>/H<sup>+</sup> exchanger regulating factor NHERF2. In *Xenopus laevis* oocytes expression of TRPV5 increases uptake of tracer  $\text{Ca}^{2+}$  and induces a  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}}$ ). In the

presence of Cl<sup>-</sup>, TRPV5 mediated  $\text{Ca}^{2+}$  entry leads to secondary activation of  $\text{Ca}^{2+}$ -sensitive Cl<sup>-</sup> channels ( $I_{\text{Cl(Ca)}}$ ). Coexpression of TRPV5 with both S<sup>422D</sup>SGK1 and NHERF2 stimulates tracer  $\text{Ca}^{2+}$  entry,  $I_{\text{Ca}}$  and  $I_{\text{Cl(Ca)}}$ . The effect of S<sup>422D</sup>SGK1 on TRPV5 and NHERF2 expressing oocytes is mimicked by SGK1 and SGK3, but not by SGK2, constitutively active T<sup>308D,S473D</sup>PKB or inactive K<sup>127N</sup>SGK1. The observations suggest that SGK1, SGK3 and NHERF2 regulate TRPV5 and are thus likely to participate in the regulation of calcium homeostasis.

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## Introduction

Maintenance of  $\text{Ca}^{2+}$  homeostasis requires the fine tuning of intestinal  $\text{Ca}^{2+}$  absorption and renal  $\text{Ca}^{2+}$  excretion [1, 2]. The epithelial  $\text{Ca}^{2+}$  channel TRPV5 [3, 4] plays a key role in both, intestinal absorption and renal

reabsorption of  $\text{Ca}^{2+}$ . The channel accomplishes  $\text{Ca}^{2+}$  uptake across the apical membrane of epithelial cells. Subsequently,  $\text{Ca}^{2+}$  is extruded via the basolateral  $\text{Ca}^{2+}$  ATPase and  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger [5]. As entry via TRPV5 is the rate limiting step in the transcellular transport of  $\text{Ca}^{2+}$ , TRPV5 activity is decisive for transepithelial transport regulation [2]. The C-terminal tail of TRPV5 contains a PDZ binding motif which may bind to the PDZ domains of NHE regulating factors NHERF1 or NHERF2 [6]. NHERF1 and NHERF2 modulate the targeting and trafficking of several proteins including TRPV4 into the plasma membrane [7-9]. TRPV5 [10] and NHERF2 [11] colocalize in principal cells. Thus, the possibility was considered that NHERF2 participates in the regulation of TRPV5 activity.

Most recent experiments disclosed a role of serum and glucocorticoid dependent kinase SGK1 in the interaction of NHE3 with NHERF2 [12, 13]. SGK1 has originally been cloned as a glucocorticoid sensitive gene from rat mammary tumor cells [14, 15]. The human isoform has been identified as a cell volume regulated gene [16]. Subsequent studies revealed the genomic regulation of SGK1 by aldosterone [17-21],  $1,25(\text{OH})_2\text{D}_3$  [22], TGF $\beta$  [23] and a variety of further cytokines [24].

SGK1 shares 80% homology with its isoforms SGK2 and SGK3 [25] and 60% homology with the protein kinase B [26]. All three isoforms SGK1-3 and protein kinase B are activated via a signaling cascade involving phosphatidylinositol 3 kinase and phosphoinositide dependent kinase PDK1 [25-28]. The activation of SGK1 requires phosphorylation at Ser422, the activation of PKB phosphorylation at Thr308 and Ser473. Replacement of those amino acids by aspartate leads to the respective constitutively active kinases,  $\text{S}^{422\text{D}}$ SGK1 [27] and  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}$ PKB [26]. Destruction of the catalytic subunit by replacement of the lysine at position 127 with asparagine leads to the inactive mutant  $\text{K}^{127\text{N}}$ SGK1 [27].

SGK1 [16], SGK2 [25], SGK3 [25] and protein kinase B [29] are expressed in a wide variety of human epithelial tissues including intestine, kidney and placenta. Thus, they may participate in the regulation of transport in those tissues. Similar to TRPV5 and NHERF2, SGK1 is expressed in principal cells of the distal nephron [30] and may well participate in the regulation of TRPV5.

The present study has been performed to test for a role of the SGK isoforms, protein kinase B and NHERF2 in the regulation of TRPV5 activity. To this end, cRNA encoding TRPV5 has been injected with or without NHERF2 and/or wild-type SGK1, SGK2, SGK3,

constitutively active  $\text{S}^{422\text{D}}$ SGK1, inactive  $\text{K}^{127\text{N}}$ SGK1, or constitutively active  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}$ PKB into *Xenopus* oocytes.

## Materials and Methods

### *Expression in Xenopus laevis oocytes*

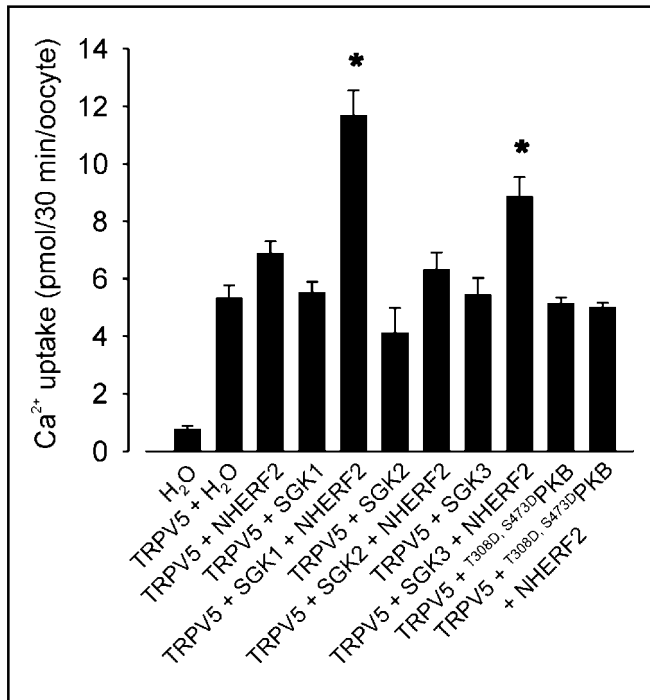
cRNA encoding rabbit TRPV5 [3] which is highly homologous to human TRPV5, human NHERF2 [13], human SGK1 [16], human SGK2 [25], human SGK3 [25], constitutively active human  $\text{S}^{422\text{D}}$ SGK1 [25], inactive human  $\text{K}^{127\text{N}}$ SGK1 [25], and constitutively active human  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}$ PKB [26] have been synthesized as described [31]. Oocytes were injected with 2.5 ng TRPV5, 7.5 ng kinase and/or 5 ng NHERF2 or NHERF2 $\Delta$ P1 or NHERF2 $\Delta$ P2 cRNA or  $\text{H}_2\text{O}$ . All experiments were performed at room temperature 3 days after injection of the respective cRNAs.

### *Tracer $\text{Ca}^{2+}$ uptake*

Calcium uptake was measured similar to what has been described earlier [32-34] with slight modifications. In detail, uptake of  $^{45}\text{Ca}^{2+}$  (ICN Biomedicals GmbH, Eschwege, Germany), delivered as  $\text{CaCl}_2$  in aqueous solution (specific activity: 0.185-1.11 TBq/g Ca) was determined 3 days after injection of oocytes with the respective cRNAs by incubating 10 - 15 oocytes at 20 °C in uptake solution containing 96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , 1 mM  $\text{BaCl}_2$ , 10  $\mu\text{M}$  methoxyverapamil, 5 mM HEPES-Tris, pH 7.4. Radioactive  $^{45}\text{Ca}^{2+}$  was added to yield a final concentration of 7  $\mu\text{Ci}/\text{ml}$ . After 30 min (within the linear range of  $^{45}\text{Ca}^{2+}$  uptake) the oocytes were washed three times in stop buffer containing 96 mM NaCl, 1 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{CaCl}_2$ , 1.5  $\text{LaCl}_3$ , 5 mM HEPES-Tris, pH 7.4. Single oocytes were then placed separately in scintillation vials and solubilized in 200  $\mu\text{l}$  10 % (w/v) sodium dodecyl sulfate. After addition of 3 ml scintillation fluid (Ultima Gold, Packard, Groningen, The Netherlands), radioactivity in the samples was measured in a liquid scintillation counter (Wallac, Freiburg, Germany).

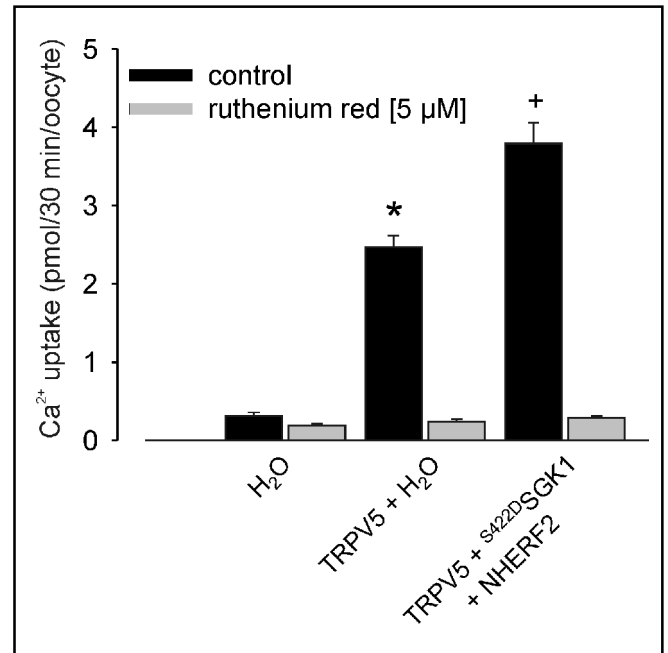
### *Voltage-clamp analysis*

In two-electrode voltage-clamp experiments currents were recorded during a 4 s linear voltage ramp from -150 mV to +50 mV. The intermediate holding potential between the voltage ramps was -50 mV. Data were filtered at 1000 Hz and recorded with MacLab digital to analog converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). The bath solution contained 96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{BaCl}_2$ , 10  $\mu\text{M}$  methoxyverapamil, 5 mM HEPES, pH 7.4 with or without 10 mM  $\text{CaCl}_2$ . Oocytes were kept in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM  $\text{NaHCO}_3$ , 0.8 mM  $\text{MgSO}_4$ , 0.3 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.4 mM  $\text{CaCl}_2$  and 5 mM HEPES, pH 7.4 supplemented with 25  $\mu\text{g}/\text{ml}$  gentamycin. The final solutions were titrated to the pH indicated using HCl or NaOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s. For direct determination of  $\text{Ca}^{2+}$  currents ( $I_{\text{Ca}}$ ), the experiments were performed with  $\text{Cl}^-$  depleted oocytes (bathed



**Fig. 1.** Stimulation of tracer  $\text{Ca}^{2+}$  entry by the combined expression of TRPV5, NHERF2 and SGK1 or SGK 3 but not of SGK2 or protein kinase B. *Xenopus laevis* oocytes were injected with either water or cRNA encoding TRPV5 with or without co-injection of SGK1, SGK2, SGK3, constitutively active T308D, S473D PKB or NHERF2 alone and combined with the mentioned kinases.  $^{45}\text{Ca}^{2+}$  uptake was measured after 30 minutes of incubation ( $n = 8-46$ ). \* denotes significant difference ( $p < 0.05$ ) between oocytes expressing TRPV5 together with either SGK1 or SGK3 and NHERF2 and oocytes expressing TRPV5 alone.

for 24 hours in  $\text{Cl}^-$  free medium) in the absence of extracellular  $\text{Cl}^-$  in the bath and KCl (3 M) filled agar bridges were used as reference electrodes to minimize liquid junction potentials. Under those experimental conditions, the currents were insensitive to the  $\text{Cl}^-$  channel blocker NPPB (100  $\mu\text{M}$ ). In the presence of  $\text{Cl}^-$  and absence of  $\text{Cl}^-$  channel inhibitors, the addition of 10 mM  $\text{CaCl}_2$  induced an inward current ( $I_{\text{Cl}(\text{Ca})}$ ) which was created by entry of  $\text{Ca}^{2+}$  and subsequent activation of  $\text{Ca}^{2+}$  sensitive  $\text{Cl}^-$  channels [4]. The peak inward current was taken as a measure for TRPV5 activity.  $I_{\text{Cl}(\text{Ca})}$  is triggered by the intracellular calcium concentration close to the cell membrane which is in turn a function of  $\text{Ca}^{2+}$  entry through the epithelial calcium channel TRPV5. Thus,  $I_{\text{Cl}(\text{Ca})}$  activity mirrors activation and inactivation kinetics of TRPV5 [35].  $I_{\text{Cl}(\text{Ca})}$  is inhibited by NPPB. Where indicated, ruthenium red was added at a concentration of 5  $\mu\text{M}$  to inhibit TRPV5 [3]. Ruthenium red further inhibits voltage-sensitive calcium channels [36], which in our experiments are already suppressed by 10  $\mu\text{M}$  methoxyverapamil. Expression and currents may vary from batch

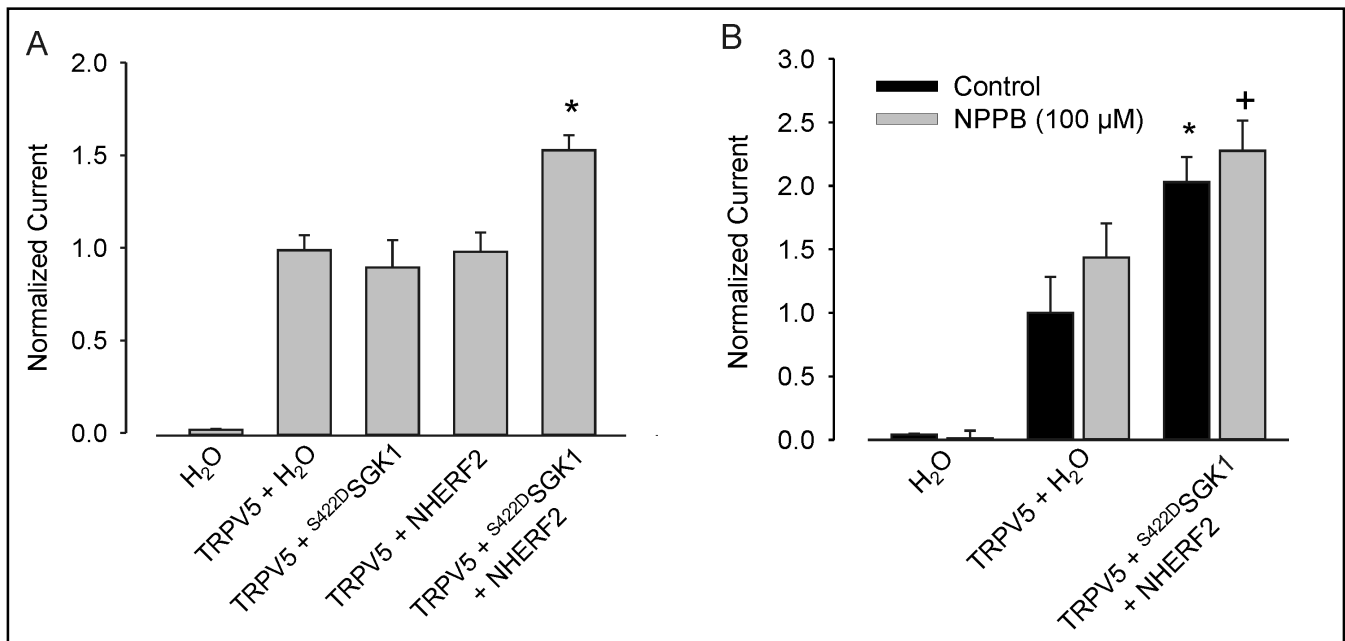


**Fig. 2.** Inhibitory effect of ruthenium red on TRPV5 activity.  $\text{Ca}^{2+}$  entry via TRPV5 with or without S422D SGK1 and NHERF2 was inhibited by addition of 5  $\mu\text{M}$  ruthenium red ( $n = 6-8$ ). *Xenopus laevis* oocytes were injected with water alone or TRPV5 with or without cRNA encoding S422D SGK1 and NHERF2. \* denotes significant difference ( $p < 0.05$ ) between oocytes expressing TRPV5 and oocytes injected with water. + denotes significant difference ( $p < 0.05$ ) between oocytes expressing TRPV5 together with S422D SGK1 and NHERF2 and oocytes expressing TRPV5 alone.

to batch. Thus, care was taken to make comparisons always within batches.

#### Detection of cell surface expression by chemiluminescence

Defolliculated oocytes were first injected with SGK1 cRNA (7.5 ng/oocyte) and/or NHERF2 cRNA (5 ng/oocyte), and one day later with TRPV5-HA cRNA which contains a HA epitope extracellularly between amino acid 376 and 377 (2.5 ng/oocyte). Oocytes were incubated with 1  $\mu\text{g}/\text{ml}$  primary rat monoclonal anti-HA antibody (clone 3F10, Boehringer, Mannheim, Germany), and 2  $\mu\text{g}/\text{ml}$  secondary, peroxidase-conjugated affinity-purified F(ab')<sub>2</sub> goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, USA). Individual oocytes were placed in 20  $\mu\text{l}$  of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer by integrating the signal over a period of 1 s. Results are given in relative light units (RLU). RLU obtained with H<sub>2</sub>O-injected



**Fig. 3.** Stimulation of  $\text{Ca}^{2+}$  currents by the combined expression of TRPV5, NHERF2 and  $\text{S}^{422\text{D}}$ SGK1. **A** TRPV5 expressing oocytes but not water injected oocytes show an inward current upon addition of 10 mM  $\text{Ca}^{2+}$  to the bath. Coexpression of TRPV5 together with  $\text{S}^{422\text{D}}$ SGK1 and NHERF2 leads to a significant increase of the current compared to oocytes expressing TRPV5 alone.  $\text{S}^{422\text{D}}$ SGK1 and NHERF2 alone do not alter TRPV5 channel activity significantly ( $n = 8-26$ ). Currents are normalized to the values obtained with oocytes expressing TRPV5 alone. \* denotes significant difference ( $p < 0.05$ ) between oocytes expressing TRPV5/ $\text{S}^{422\text{D}}$ SGK1 together with NHERF2 and

oocytes injected with TRPV5 alone. **B** The inward current into TRPV5 expressing oocytes in the absence of  $\text{Cl}^-$  induced by high extracellular  $\text{Ca}^{2+}$  is not sensitive to the chloride channel blocker NPPB. Currents are normalized to the values obtained with oocytes expressing TRPV5 alone in the absence of NPPB. \* denotes significant difference ( $p < 0.05$ ) between oocytes expressing TRPV5,  $\text{S}^{422\text{D}}$ SGK1 together with NHERF2 and oocytes expressing TRPV5 alone. + denotes significant difference between oocytes expressing TRPV5,  $\text{S}^{422\text{D}}$ SGK1 together with NHERF2 and oocytes expressing TRPV5 alone under continuous presence of 100  $\mu\text{M}$  NPPB ( $n = 5-12$ ).

oocytes (control oocytes) were subtracted from the values obtained with oocytes injected with TRPV5-HA alone or together with SGK1 and/or NHERF2.

#### Statistics

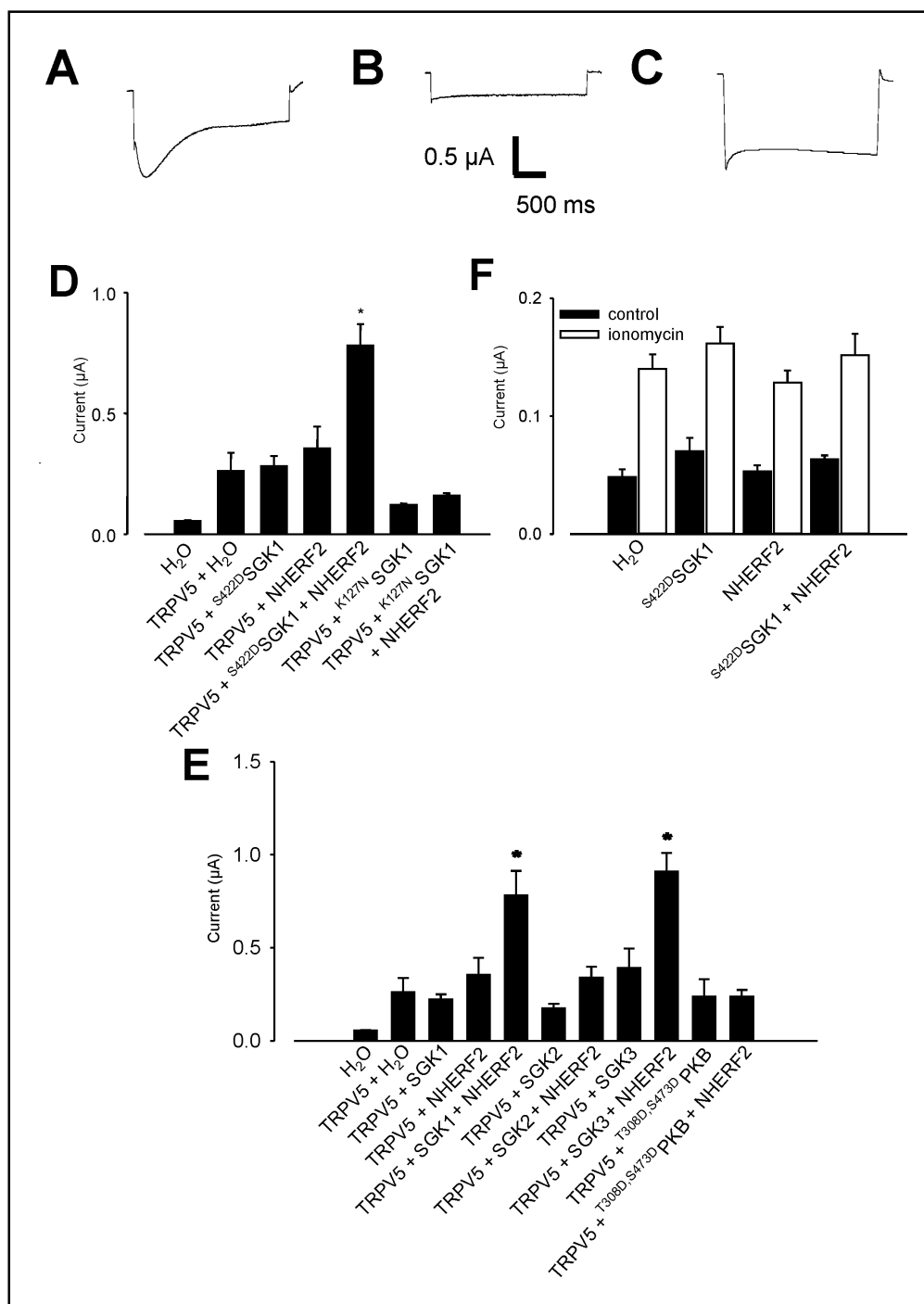
Data are provided as means  $\pm$  SEM,  $n$  represents the number of oocytes investigated. All experiments were repeated with at least 3 batches of oocytes from different frogs; in all repetitions qualitatively similar data were obtained. All data were tested for significance using ANOVA and only results with  $P < 0.05$  were considered statistically significant.

## Results

As evident from Fig. 1, uptake of radioactive  $\text{Ca}^{2+}$  ( $^{45}\text{Ca}^{2+}$ ) into *Xenopus* oocytes was increased some 7 fold by expression of TRPV5. The increase of  $\text{Ca}^{2+}$  uptake reflects the  $\text{Ca}^{2+}$  transporting capacity of TRPV5.

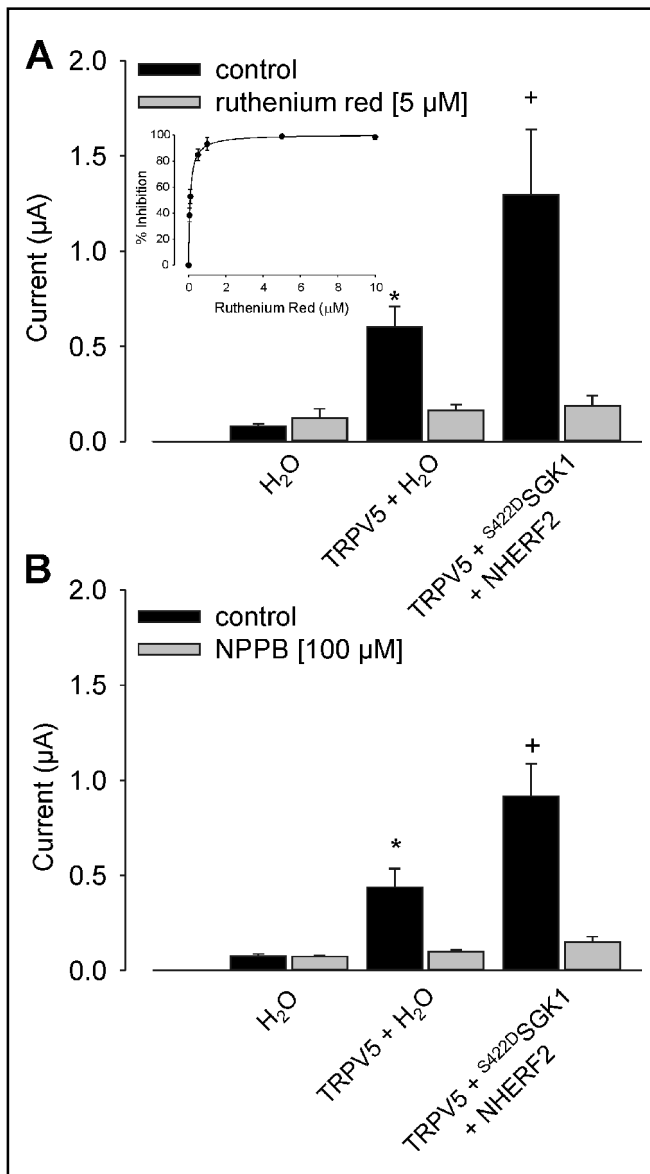
Additional expression of either NHERF2, SGK1, SGK2, SGK3 or constitutively active  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}\text{PKB}$  alone did not further stimulate  $^{45}\text{Ca}^{2+}$  entry. However, expression of TRPV5 together with both SGK1 and NHERF2 resulted in significant further stimulation of  $\text{Ca}^{2+}$  uptake. In the absence of TRPV5, expression of SGK1 and NHERF2 did not increase  $\text{Ca}^{2+}$  uptake (not shown). Thus, coexpression of SGK1 and NHERF2 increased  $\text{Ca}^{2+}$  uptake by stimulation of TRPV5. Similarly, the coexpression of TRPV5 together with SGK3 and NHERF2 increased  $\text{Ca}^{2+}$  uptake. Both kinases stimulated TRPV5, an effect requiring the presence of NHERF2. In contrast, the coexpression of neither SGK2 nor  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}\text{PKB}$  significantly modified the  $\text{Ca}^{2+}$  uptake into oocytes expressing TRPV5 and NHERF2. Thus, SGK1 and SGK3, but not SGK2 or  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}\text{PKB}$  stimulate TRPV5 in the presence of NHERF2.

**Fig. 4.** TRPV5 mediated calcium currents indirectly activate an endogenous chloride conductance ( $I_{Cl(Ca)}$ ). **A - C** In TRPV5 expressing *Xenopus laevis* oocytes, hyperpolarization from -50 mV to -110 mV in the presence of 10 mM  $Ca^{2+}$  triggered a rapidly activating, slowly and partially inactivating inward current (A) while in water injected cells hyperpolarization does not increase any voltage-dependent current (B). Elevation of cytosolic  $Ca^{2+}$  by addition of the  $Ca^{2+}$  ionophore ionomycin (10  $\mu$ M) led to the stimulation of a non inactivating current (C). **D - E**  $I_{Cl(Ca)}$  was stimulated by coexpression of TRPV5 together with NHERF2 and either  $S^{422D}$ SGK1 (D), SGK1 or SGK3 (E). Coexpression of TRPV5 with either NHERF2,  $S^{422D}$ SGK1, SGK1 or SGK3 alone did not significantly enhance  $I_{Cl(Ca)}$  (D, E) ( $n = 19-26$ ). **F** The ionomycin induced endogenous  $Cl^-$  current was not significantly enhanced by expression of  $S^{422D}$ SGK1, NHERF2 or both ( $n = 6$ ). \* denotes significant difference ( $p < 0.05$ ) between oocytes expressing TRPV5 together with either  $S^{422D}$ SGK1, SGK1 or SGK3 and NHERF2 and oocytes expressing TRPV5 alone.

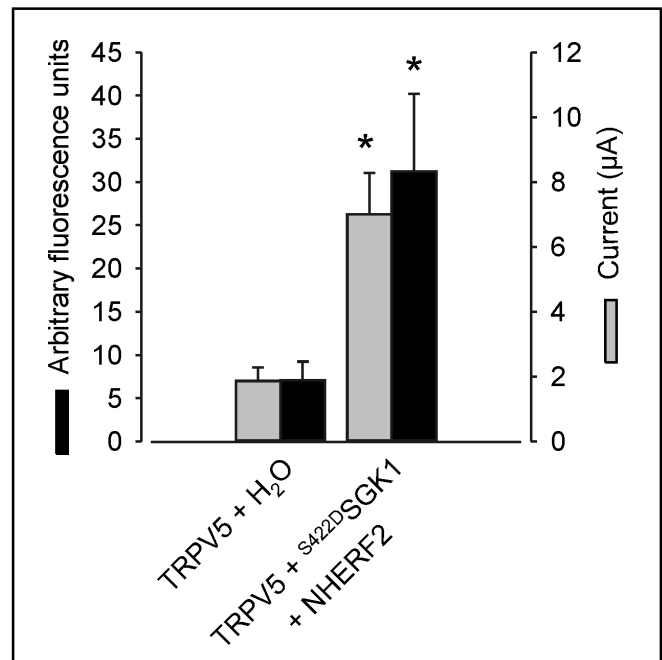


Previously, TRPV5 has been shown to be inhibited by ruthenium red [32], a polycationic dye. Thus, the effect of ruthenium red on  $Ca^{2+}$  uptake was tested. As shown in Fig. 2, 5  $\mu$ M ruthenium red completely inhibited  $Ca^{2+}$  uptake in TRPV5 expressing oocytes with or without additional expression of  $S^{422D}$ SGK1 and NHERF2.

Further studies have been performed to determine the influence of  $S^{422D}$ SGK1 on TRPV5 mediated currents. As reported earlier, the entry of  $Ca^{2+}$  triggers  $Ca^{2+}$  sensitive  $Cl^-$  channels [35]. To determine the  $Ca^{2+}$  current directly, the  $Cl^-$  current had to be suppressed. Therefore, the oocytes were bathed in  $Cl^-$  free extracellular solution



**Fig. 5.** Inhibition of the TRPV5 induced current  $I_{Cl(Ca)}$  by ruthenium red and NPPB. *Xenopus laevis* oocytes were injected with water alone or TRPV5 with or without cRNA encoding S<sup>422D</sup>SGK1 and NHERF2. **A** Inhibition of  $I_{Cl(Ca)}$  by application of 5 μM ruthenium red (n = 7). **B** Inhibition of  $I_{Cl(Ca)}$  by application of 100 μM NPPB (n = 5-8). Insert in A depicts dose-dependent inhibition of TRPV5 activity. \* denotes significant difference (p < 0.05) between *Xenopus* oocytes expressing TRPV5 and oocytes injected with water. + denotes significant difference (p < 0.05) between *Xenopus* oocytes expressing TRPV5 together with S<sup>422D</sup>SGK1 and NHERF2 and oocytes expressing TRPV5 alone.



**Fig 6.** TRPV5 currents and cell surface abundance are similarly increased by coexpression of S<sup>422D</sup>SGK1 and NHERF2. *Xenopus laevis* oocytes were injected with water or cRNA encoding TRPV5 alone or with S<sup>422D</sup>SGK1 and wild-type NHERF2. The combined expression of S<sup>422D</sup>SGK1 and wild-type NHERF2 increases TRPV5 channel activity and cell surface abundance. \* indicates significant difference (p < 0.05) between expression of TRPV5 alone and of TRPV5 coexpressed with S<sup>422D</sup>SGK1 and NHERF2 (n = 20-29).

for 24 hours. As shown in figure 3, coexpression of TRPV5 together with S<sup>422D</sup>SGK1 and NHERF2 led to a significant increase of the current induced by addition of 10 mM CaCl<sub>2</sub>. In contrast, the current was not increased by coexpression of TRPV5 with either NHERF2 or S<sup>422D</sup>SGK1 alone (Fig. 3A).  $I_{Ca}$  was insensitive to the Cl<sup>-</sup> channel inhibitor NPPB (Fig. 3B).

In the presence of Cl<sup>-</sup> the Ca<sup>2+</sup> entry through TRPV5 stimulated Ca<sup>2+</sup> sensitive Cl<sup>-</sup> channels leading to the appearance of a large Cl<sup>-</sup> current ( $I_{Cl(Ca)}$ ). In TRPV5-expressing oocytes, hyperpolarization from -50 mV to -110 mV in the presence of 10 mM Ca<sup>2+</sup> triggered a rapidly activating, slowly and partially inactivating inward current (Fig. 4A). The transiently activating current was only present in *Xenopus* oocytes expressing TRPV5. In water injected oocytes increase of cytosolic Ca<sup>2+</sup> activity by addition of Ca<sup>2+</sup> ionophore ionomycin (10 μM) led to the

stimulation of a non inactivating current (Fig. 4C). Fig. 4B depicts a recording from a non-injected oocyte. Similar to tracer the  $\text{Ca}^{2+}$  uptake and  $I_{\text{Ca}}$ ,  $I_{\text{Cl(Ca)}}$  was stimulated by coexpression of TRPV5 together with NHERF2 and either,  $\text{S}^{422\text{D}}$ SGK1 (Fig. 4D), SGK1 (Fig. 4E) or SGK3 (4E). Coexpression of TRPV5 with either NHERF2,  $\text{S}^{422\text{D}}$ SGK1, SGK1 or SGK3 alone did not significantly enhance  $I_{\text{Cl(Ca)}}$ . Moreover, neither SGK2 nor  $\text{T}^{308\text{D},\text{S}^{473\text{D}}}$ PKB stimulated  $I_{\text{Cl(Ca)}}$ , even upon NHERF2 coexpression (Fig. 4E). Thus,  $I_{\text{Cl(Ca)}}$  was enhanced by SGK1 and SGK3 only in the presence of NHERF2. In oocytes not expressing TRPV5, the  $\text{Ca}^{2+}$  sensitive  $\text{Cl}^-$  current could be activated by the  $\text{Ca}^{2+}$  ionophore ionomycin (10  $\mu\text{M}$ ). As shown in Fig. 4F, the ionomycin induced  $\text{Cl}^-$  current was not significantly enhanced by expression of  $\text{S}^{422\text{D}}$ SGK1, NHERF2 or both. Thus, the increase of  $I_{\text{Cl(Ca)}}$  following coexpression of  $\text{S}^{422\text{D}}$ SGK1 and NHERF2 was not due to upregulation of the  $\text{Cl}^-$  channel but due to upregulation of TRPV5.

As illustrated in Fig. 5,  $I_{\text{Cl(Ca)}}$  was abolished by ruthenium red (Fig. 5A) and by the  $\text{Cl}^-$  channel blocker NPPB (Fig. 5B).

To discriminate whether increased  $\text{Ca}^{2+}$  fluxes and  $\text{Ca}^{2+}$  currents upon coexpression of SGKs and NHERF2 are due to modulation of TRPV5 gating kinetics or induced transport of TRPV5 to the cell membrane, we quantified cell surface expression of the channel and the peak current voltage. As depicted in Fig. 6, TRPV5 currents and cell surface expression of the channel protein were similarly increased by coexpression of  $\text{S}^{422\text{D}}$ SGK1 and NHERF2.

## Discussion

The present observations clearly demonstrate that expression of TRPV5 induces  $\text{Ca}^{2+}$  influx allowing the cellular accumulation of tracer  $\text{Ca}^{2+}$  and generating a  $\text{Ca}^{2+}$  current. In the presence of  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$  entry through TRPV5 generates further currents [3] by activation of endogenous  $\text{Ca}^{2+}$  sensitive  $\text{Cl}^-$  channels [37].

More importantly, the present observations disclose a completely novel mechanism regulating TRPV5 activity, i.e. the regulation by two members of the serum and glucocorticoid inducible kinase family and the NHE regulating factor NHERF2. The effect of SGK1 depends on an intact catalytic subunit, as the inactive mutant  $\text{K}^{127\text{N}}$ SGK1 does not influence TRPV5 even in the presence of NHERF2. Thus, the kinases are obviously effective through phosphorylation of target proteins. The

stimulating effect is shared by SGK1 and SGK3, but not by SGK2 or PKB. The TRPV5-stimulating capacity correlates with the ability of the two kinases to bind NHERF2 [6]. Expressed wild type SGK1 and SGK3 are not constitutively active but require activation. The kinases are activated through a signaling pathway involving phosphatidylinositol 3 kinase (PI3-K) and phosphoinositide dependent kinase (PDK) [27]. Obviously, the activation of the kinases is not limiting in oocytes, as expression of wild type SGK3 is similarly effective compared to  $\text{S}^{422\text{D}}$ SGK1. In earlier studies, wild type SGK1 has similarly been shown to be effective [38]. In contrast to SGK1 and SGK3, SGK2 does not bind NHERF2 [6] and is, according to the present study, not able to stimulate TRPV5. PKB is similarly not able to stimulate TRPV5.

A well known function of SGK1 is its participation in the regulation of the epithelial  $\text{Na}^+$  channel ENaC [17, 18, 20, 39-45]. SGK1 is effective by increasing the abundance of the ENaC protein within the cell membrane [30, 39, 45]. Most recently, SGK1 has been shown to upregulate the voltage gated  $\text{K}^+$  channel Kv1.3 [46-48], the epithelial  $\text{Na}^+/\text{H}^+$  exchanger NHE3 [6] and the renal epithelial  $\text{K}^+$  channel ROMK1 [49]. Similar to SGK-dependent regulation of TRPV5, the regulation of ROMK1 by SGK1 has been shown to depend on NHERF2 [49].

In contrast to the regulation of TRPV5, NHE3 and ROMK1, the regulation of ENaC or Kv1.3 does not require the participation of NHERF2. SGK1 stimulates ENaC by phosphorylating the ubiquitin protein ligase Nedd4-2 in a PY motif-dependent manner [50, 51]. The phosphorylation impedes the binding of Nedd4-2 to ENaC [50]. The ENaC sequence does not include a PDZ motif, suggesting that NHERF2 can not directly interact with ENaC.

As NHERF2 regulates both TRPV5 and NHE3, it may participate in the link between TRPV5 activity and acid base balance. TRPV5 displays an exquisite  $\text{H}^+$  sensitivity [1, 3, 22]. Its activity is markedly reduced by lowering of the ambient pH. The sensitivity of renal tubular  $\text{Ca}^{2+}$  transport to  $\text{H}^+$  is of physiological significance, as on the one hand mineralization of bone depends on the deposition of highly alkaline  $\text{Ca}^{2+}$  salts, [52] and on the other, precipitation of  $\text{Ca}^{2+}$  phosphate salts is favoured by alkalization of urine [53]. Most recently, NHERF2 has been shown to direct the signaling of the PTH receptor [54]. Since PTH is a key regulator of TRPV5 [1], this effect may similarly contribute to the regulation of TRPV5. However, according to the present observations, NHERF2 alone is unable to activate TRPV5 but requires



the additional action of SGK1 or of one of its isoforms. SGK1 expression is highly variable, as it is upregulated by glucocorticoids [15], aldosterone [17-21], cell shrinkage [16] and a wide variety of additional factors [24]. Notably, SGK1 is under transcriptional control of  $1,25(\text{OH})_2\text{D}_3$  [22]. It is thus tempting to speculate that SGK1 participates in the regulation of TRPV5 by  $1,25(\text{OH})_2\text{D}_3$  and PTH. Activation of SGK1 can be accomplished by insulin and IGF1 through PI3 kinase and PDK1 [27, 28]. SGK1 integrates the signals coming from genomic regulators on the one hand and insulin or IGF1 on the other [55]. Thus, activation of SGK1 may contribute to the stimulating effect of both,  $1,25(\text{OH})_2\text{D}_3$  and IGF1 on intestinal  $\text{Ca}^{2+}$  absorption [56].

In conclusion, TRPV5 is the target of a complex regulatory mechanism involving both, the NHE regulating factor NHERF2 and the serine/threonine kinases SGK1 and 3. The concerted action of NHERF2 and the kinases markedly up regulates the activity of this key channel in the regulation of  $\text{Ca}^{2+}$  homeostasis.

## Abbreviations

SGK (serum and glucocorticoid inducible kinase); NHERF (sodium hydrogen exchanger regulating factor); Nedd (Neuronal cell expressed developmentally downregulated ubiquitin ligase).

## Acknowledgements

The authors acknowledge the technical assistance of B. Noll and the meticulous preparation of the manuscript by Lejla Subasic and Tanja Loch. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-4 and La 315/5-1, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research) 01 KS 9602 and the National Institutes of Health Grant DK-44484. PC is supported by the UK Medical Research Council and The Royal Society. RB is supported by grants from the Dutch Organization of Scientific Research (Zon-Mw 016.006.001, Zon-Mw 902.18.298, NWO-ALW 810.38.004, NWO-ALW 805.09.042).

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